



UNIVERSITI PUTRA MALAYSIA

**CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS
ISOLATED IN MALAYSIA FOR THE DEVELOPMENT OF
DIAGNOSTIC TOOLS**

PHONG SU FUN

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DIAGNOSTIC TOOLS**

By

PHONG SU FUN

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia in Fulfilment of Requirement for the
Degree of Doctor of Philosophy**

May 2002



**Dedicated with love and gratitude
to:**

**My husband Wesley Voon, parents,
daughter (Wynn timer) and son (Wingates)**

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

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May 2002

Chairman: Mohd Hair-Bejo, Ph.D

Faculty: Veterinary Medicine

The P97/302 field outbreak isolate was identified as a very virulent infectious bursal disease virus (vvIBDV) of serotype 1, based on the conventional and molecular characterization methods. The high mortality, gross and histopathological lesions observed during the outbreak and in the experimental infected SPF chickens induced by P97/302 IBDV isolate were characteristics of vvIBDV strain reported previously. The sequence of P97/302 isolate has amino acid substitutions at 222(A), 256(I), 294(I) and 299(S) similar to other reported vvIBDV. This isolate does not have 249(K) and 254(S) amino acid residues which have been reported to be present in variant strains. The amino acid residues of the P97/302 at the two hydrophilic regions and the serine-rich heptapeptide region are the same as reported for vv strains of UK661, HK46 and OKYM. The P97/302 IBDV isolate can be digested with *Taq*1, *Acc*1, *Sty*1, *Spe*1 enzymes and not with



Sac1 as reported for other vv IBDV. This isolate is most homologous to the reported vv IBDV strains especially UK661. Phylogenetic analysis based on the nucleotide sequence of the hypervariable region revealed that P97/302 IBDV isolate can be clustered with the vvIBDV strains and is distinct from classical, variant and attenuated strains. Using this P97/302 IBDV local isolate, the study has successfully developed three diagnostic tools for antibody and antigen detection. They are the indirect, double antibody sandwich (DAS) and reverse-transcription (RT) nested polymerase chain reaction (PCR) enzyme-linked immunoassay assay (ELISA). The developed indirect and DAS ELISA are based on the use of regression equation line which generated from the standard curves to measure IBD antibody titre. They are highly significant correlated ($p < 0.01$) when compared with IDEXX commercial ELISA. They are more advantage than the commercial kits whereby the local isolate was used for the ELISA coating which was able to enhance the accuracy to predict the protection to IBD. The developed DAS ELISA for antigen detection is also highly specific. The developed RT nested PCR was highly specific and ten times more sensitive when compared to conventional RT/PCR. The RT nested PCR ELISA method was also highly specific and hundred times more sensitive when compared to conventional RT/PCR with agarose gel electrophoresis detection method. It was concluded that the P97/302 local isolate was vvIBDV strain and the developed indirect, DAS and RT nested PCR ELISA are potentially used for monitoring and screening large number of samples for antibody and antigen detection from chicken flocks.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Doktor Falsafah

**PENCIRIAN VIRUS PENYAKIT BURSA BERJANGKIT YANG
DIPEROLEHI DARI MALAYSIA UNTUK MEMBUAT ALAT-ALAT
DIAGNOSA**

Oleh

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P97/302 strain dari ladang ayam telah dikenalpasti sebagai serotip 1 virus penyakit bursa berjangkit yang amat virulen (vvIBDV) berdasarkan pencirian cara konvensional dan molekul. Kadar kematian yang tinggi, lesi-lesi patologi dan histopatologi semasa outbreak dan pada ayam bebas-patogen khusus (SPF) telah mencirikannya sebagai vvIBDV seperti yang pernah dilaporkan. Jujukan P97/302 mempunyai penggantian asid amino di kedudukan 222(A), 256(I), 294(I) dan 299(S) seperti yang dilaporkan pada vvIBDV yang lain. P97/302 tidak mempunyai asid amino residu di kedudukan 249(K) dan 254(S) seperti yang dilaporkan pada strain varian. Ia mempunyai asid amino residu yang sama dengan vvIBDV yang lain iaitu UK661, HK46 dan OKYM, di dua kawasan hidrofilik dan kawasan heptapeptid yang kaya dengan serine. Ia boleh dicernakan oleh enzim pembatas *Taq1*, *Acc1*, *Sty1*, *Spe1* dan tidak pula oleh *Sac1* sepertimana

yang dilaporkan pada vvIBDV yang lain. P97/302 sangat homolog dengan vvIBDV lain yang dilaporkan, terutamanya dengan UK661. Analisis filogenesis berdasarkan kawasan hiper boleh ubah jujukan nukleotid menunjukkan strain ini sekumpulan dengan vvIBDV dan berlainan kumpulan dengan strain-strain klasik, varian dan attenuated. Dengan menggunakan P97/302 IBDV, kajian ini telah berjaya membuat tiga jenis alat diagnosa untuk mengesan antibodi dan antigen. Alat-alat ini ialah indirect, double antibodi sandwich (DAS) dan reverse-transkripsi (RT) nested reaksi rantai polimerasi (PCR) asai immunoerap terangkai enzim (ELISA). Indirect dan DAS ELISA ini adalah berdasarkan penggunaan formula regresi yang dibentuk dari garis standard untuk mengukur antibodi IBD titer. Alat-alat ini menunjukkan kolerasi yang sangat ketara ($p < 0.01$) apabila dibandingkan dengan komersial ELISA IDEXX. Alat-alat ini lebih baik daripada ELISA komersial kerana strain tempatan digunakan untuk melapis ELISA yang akan meningkatkan ketepatan meramal perlindungan daripada IBD. DAS ELISA juga sangat spesifik untuk mengesan antigen. RT nested PCR juga didapati sangat spesifik dan menunjukkan sepuluh kali lebih sensitif apabila dibandingkan dengan konvensional RT/PCR. RT nested PCR ELISA pula didapati sangat spesifik dan telah menunjukkan seratus kali lebih sensitif apabila dibandingkan dengan konvensional RT/PCR yang menggunakan agarose gel elektroforesi. Kesimpulannya, P97/302 strain adalah vvIBDV dan indirect, DAS dan RT nested PCR ELISA ini adalah berpotensi untuk mengkaji dan menyiasat sampel antibodi dan antigen dari kelompok ayam.

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I certify that an Examination Committee met on 2nd May 2002 to conduct the final examination of Phong Su Fun on her Doctor of Philosophy thesis entitled “ Characterization of Infectious Bursal Disease Virus Isolated in Malaysia for the Development of Diagnostic Tools” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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I hereby declare that the thesis is based on my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



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TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	v
ACKNOWLEDGEMENTS	vii
APPROVAL SHEETS	viii
DECLARATION FORM	x
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xx
 CHAPTER	
I INTRODUCTION.....	1
 II LITERATURE REVIEW.....	12
Infectious Bursal Disease Virus (IBDV).....	12
Resistance to Chemical and Physical Agents.....	15
Antigenic and Virulence Variation.....	15
Clinical Signs and Gross Pathology.....	19
Histopathology.....	21
Transmission.....	22
Immunosuppression.....	23
Embryonated Chickens Eggs.....	25
Tissue Culture.....	26
Replication Efficiency.....	26
Diagnosis.....	27
Prevention and Control.....	32
Enzyme-Linked Immunosorbent Assay (ELISA).....	36
Indirect Antibody and Antigen ELISA.....	39
Sandwich Antigen-Capture and Antibody-Capture ELISA.....	40
Polymerase Chain Reaction	44
Nested PCR.....	45
PCR Product Detection Method.....	47
PCR ELISA Detection.....	48
Primer Dimerization.....	49
Phylogenetic Analysis of IBDV.....	51



III	ISOLATION AND IDENTIFICATION OF LOCAL ISOLATE INFECTIOUS BURSAL DISEASE VIRUS.....	53
	Introduction.....	53
	Materials and Methods.....	58
	IBD Field Outbreak.....	58
	Processing of Sample.....	59
	Embryonated Chicken Eggs.....	59
	CAM Preparation.....	59
	IBDV Inoculation.....	60
	IBDV Purification.....	60
	IBDV Detection.....	61
	Transmission Electron Microscopy.....	61
	Chicken Embryo Fibroblast (CEF) Tissue Culture.....	62
	CEF Preparation.....	62
	IBDV Isolation.....	64
	IBDV Identification.....	64
	Immunoperoxidase Test.....	64
	Immunofluorescent Test.....	65
	SPF Chickens.....	66
	Experiment 1.....	66
	Experiment 2.....	66
	Detection and Identification of IBDV.....	67
	Transmission Electron Microscopy.....	67
	Immunofluorecence Test.....	68
	Results.....	68
	IBD Field Outbreak.....	68
	Embryonated Chicken Eggs.....	69
	CAM Purification and IBDV Detection.....	69
	Chicken Embryo Fibroblast Tissue Culture.....	70
	IBDV Identification.....	70
	SPF Chickens.....	70
	Experiment 1.....	70
	Experiment 2.....	71
	Histopathological Finding.....	71
	Detection and Identification of the IBDV.....	72
	Transmission Electron Microscopy.....	72
	Bursa Impression Smear.....	72
	IBD Antibody Titre.....	72
	Discussion.....	76

IV	MOLECULAR CHARACTERIZATION OF VP2 IBDV LOCAL ISOLATE.....	83
	Introduction	83
	Materials and Methods	87
	Sample Preparation.....	87
	Virus Purification	88
	RNA Extraction	88
	Determination of RNA Concentration and Purity.....	89
	Primer Design.....	90
	cDNA Synthesis and PCR Amplification.....	91
	Determination of PCR Product Concentration and Purity.....	92
	Agarose Gel Electrophoresis	93
	Ethidium Bromide Staining.....	93
	Extraction and Purification of PCR product.....	94
	Cloning of the Full Length VP2 Gene.....	95
	TOPO Cloning Reaction.....	95
	One Shot™ Chemical Transformation Reaction.....	95
	Subculture and Analysis of Positive Colonies.....	96
	Preparation of Stock Culture.....	96
	Plasmid Extraction and Purification.....	96
	Plasmid Concentration and Purity.....	98
	Restriction Enzyme Digestion Analysis.....	98
	Cycle and DNA Sequencing	98
	Sequence Assembly and Analysis.....	99
	Phylogenetic Analysis.....	100
	Results.....	100
	Virus Purification.....	100
	VP2 Gene Amplification	100
	Analysis of Recombinant Plasmid.....	101
	Nucleotides and Amino Acids Sequence Analysis of VP2 Gene.....	101
	Restriction Map of VP2 Gene.....	103
	Phylogenetic Analysis.....	103
	Discussion.....	118
V	DEVELOPMENT OF INDIRECT AND DOUBLE ANTIBODY SANDWICH (DAS) ELISA.....	123
	Introduction.....	123
	Materials and Methods.....	128
	Production of Chicken Hyperimmune Serum (HIS)	128
	Preparation of IBDV.....	128
	Preparation of Chicken Hyperimmune Serum (HIS)	128
	Chicken HIS Evaluation.....	129

Production of Rabbit Hyperimmune Serum.....	130
Rabbit HIS Purification.....	131
Preparation and Treatment of IBD Antigen for Coating.....	132
Antigen Preparation for DAS ELISA.....	133
Choice of Solid Phase.....	133
ELISA Optimization.....	133
Indirect ELISA.....	133
DAS ELISA.....	134
Standard Curve of Indirect and DAS ELISA.....	135
Positive-Negative Threshold (PNT) for Indirect and DAS ELISA.....	136
ELISA Procedure for Antibody Detection.....	137
Indirect ELISA.....	137
DAS ELISA.....	138
DAS ELISA for Antigen Detection.....	138
Evaluation of Indirect and DAS ELISA for Antibody Detection.....	139
Evaluation of DAS ELISA for Antigen Detection.....	141
Results.....	141
Chicken Hyperimmune Serum Production.....	141
Choice of Solid Phase.....	142
Indirect ELISA Optimization.....	142
Standard Curve of Indirect ELISA.....	143
DAS ELISA Optimization.....	143
Standard Curve of DAS ELISA.....	144
Determination of Positive-Negative Threshold (PNT)...	144
Evaluation of Indirect and DAS ELISA.....	145
DAS ELISA for Antigen Detection.....	146
Discussion.....	161
 VI DEVELOPMENT OF RT NESTED PCR ELISA..	 169
Introduction.....	169
Materials and Methods.....	173
Primers Design	173
Sample Preparation and dsRNA Extraction.....	173
cDNA Synthesis and First PCR Amplification.....	174
Second PCR Amplification.....	175
Optimization of PCR ELISA.....	175
Sensitivity Studies.....	176
Specificity Determination.....	177
Analysis of PCR Products.....	177
Agarose Gel Electrophoresis.....	177
Colorimetric Detection (ELISA) of PCR Products	177

Results.....	178
RT Nested PCR Optimization.....	178
Optimal Condition of RT Nested PCR ELISA.....	179
Sensitivity Determination of RT Nested PCR.....	180
Specificity Evaluation of RT Nested PCR	180
Sensitivity Determination of RT Nested PCR ELISA	
Detection Method.....	181
Specificity Determination of RT Nested PCR ELISA	
Detection Method.....	181
Discussion.....	190
VII GENERAL DISCUSSION AND CONCLUSION.....	195
Future Prospects and Suggestions.....	203
BIBLIOGRAPHY.....	205
APPENDICES.....	234
A Chemicals and Reagents.....	234
B Buffer and Media.....	236
C Working Cell Suspension.....	239
D Tables.....	240
E Restriction Mapping.....	243
F Determination of Protein Concentration.....	247
VITA.....	248
PUBLICATIONS.....	249

LIST OF TABLES

Table		Page
4.1	Primers used to amplify the VP2 gene (1.35kb).....	90
4.2	Primers used to sequence the VP2 gene of P97/302 IBDV isolate.....	91

LIST OF FIGURES

Figure		Page
3.1	IBD field outbreak. (a) P97/302 IBDV infected chickens were weak, depressed, inappetance and showed ruffled feather, (b) purified P97/302 IBDV isolated from CAM samples under TEM examination (Negative staining, x80 000).....	73
3.2	Infected SPF bursa of Fabricius. (a) Acute necrotizing bursitis (HE, x10), (b) cluster of P97/302 IBDV particles in the cytoplasm of lymphoid cell under TEM examination (x 20 000).....	74
3.3	IFA of the bursa impression smear from SPF chicken, (a) negative control (x10), (b) IBDV infected bursa of Fabricius was stained in bright yellow in colour (x10).....	75
4.1	RT-PCR product of the 1.35kb VP2 gene of P97/302 IBDV...	104
4.2	PCR screening on ten white colonies. Nine colonies were positive for 1.35kb of the VP2 gene insert.....	105
4.3	Purified plasmids digested with <i>EcoR</i> I enzyme.....	106
4.4	Nucleotide sequence and translation of amino acid of P97/302 IBDV isolate	107
4.5	Nucleotide sequence alignment of P97/302 IBDV isolate.....	109
4.6	Amino acid sequence alignment of P97/302 IBDV isolate.....	115
4.7	The phylogenetic tree was constructed based on nucleotide sequence of the hypervariable region of VP2 of IBDV isolates, displaying the phylogenetic relationships of the P97/302 IBDV isolate and other published IBDV strains.....	117
5.1	Hyperimmune serum production in SPF chickens. (a) Mean antibody titre, (b) mean antibody titre in Log ₁₀ vs age (weeks) of chickens.....	147
5.2	Choice of solid phase. (a) Indirect ELISA and (b) DAS ELISA.....	148

5.3	The absorbance value of serial dilution of HIS in different concentrations of antigen (1:10, 1:50 or 1:100) for coating the indirect ELISA.....	149
5.4	The absorbance value of serially diluted HIS in different concentrations of BSA (1, 2 or 5%) as blocking agent in the indirect ELISA.....	150
5.5	The absorbance value of the indirect ELISA in different concentrations of conjugate (1:1000, 1:5000 or 1: 10 000) and substrate incubation times (5 to 30 Minutes).....	151
5.6	Standard curve of indirect ELISA.....	152
5.7	The absorbance value of serially diluted HIS in different concentrations of antibody (1:10, 1:50 or 1:100) for coating the DAS ELISA.....	153
5.8	The absorbance value of serially diluted HIS in different concentrations of BSA (1, 2 or 5%) as blocking agent in the indirect ELISA.....	154
5.9	The absorbance value of the DAS ELISA in different concentrations of conjugate (1:1000, 1:5000 or 1: 10000) and substrate incubation time (5 to 30 Minutes).....	155
5.10	The standard curve of DAS ELISA.....	156
5.11	The mean antibody titres of broiler chickens from Farm A which were evaluated by IDEXX, indirect and DAS ELISA...	157
5.12	Mean antibody titre of broiler chickens from Farm B which was evaluated by IDEXX, indirect and DAS ELISA.....	158
5.13	The mean antibody titres of SPF chickens which were evaluated by IDEXX, indirect and DAS ELISA.....	159
5.14	The absorbance value of the five IBDV isolates (P97/302, P97/61, P94/284, P94/273 and P99/551), three IBDV vaccines strains (Cu-1M, LC75 and D78), NDV, CAV, negative control bursa and cut-off value of the DAS ELISA for antigen detection.....	160

6.1	The absorbance values of serials dilution of PCR product in different streptavidin concentrations (1ug/ml, 5ug/ml or 10ug/ml) for PCR ELISA coating.....	182
6.2	Checkerboard titration between peroxidase anti-digoxigenin conjugate and dilution of PCR product.....	183
6.3	The effect of incubation time of the enzyme conjugate substrate complex on absorbance value.....	184
6.4	Sensitivity determination. (a) RT nested PCR compared to, (b) conventional RT/PCR.....	185
6.5	Specificity determination of RT nested PCR.....	186
6.6	Sensitivity determination, (a) PCR ELISA compared to (b) agarose gel detection method.....	187
6.7	Sensitivity determination, (a) PCR ELISA compared to (b) agarose gel detection method.....	188
6.8	Specificity determination of RT nested PCR ELISA.....	189

LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-di(3-ethyl)benzthiazoline sulphononic acid
AGPT	Agar gel precipitin test
ASA	5-Aminosalicylic acid
BGM	Baby grivet monkey kidney
BHK	Baby hamster kidney
bp	Basepair
BSA	Bovine serum albumin
Ca	Calcium
CAM	Chorioallantoic membrane
CAV	Chicken anaemia virus
cDNA	Complementary deoxyribonucleic acid
CEB	Chicken embryo bursal cell
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
CEP	Cytopathic effect
°C	Degree Celsius
d-	Deoxy
DAS	Double antibody sandwich
dd	Dideoxy
DEPC	Diethyl pyrocarbonate
DH ₂ O	Distilled water
DIG	Digoxigenin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double strand
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme-immuno-assay
EID ₅₀	Embryo infective dose fifty
ELISA	Enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HCl	Hydrochloric acid
HIS	Hyperimmune serum
HSV	Herpes simplex virus
H ₂ O ₂	Hydrogen peroxide
HRP	Horseradish peroxidase
h _v	Hypervariable
IB	Infectious bronchitis



IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFA	Immunofluorescent test
Ig G	Immunoglobulin G
IPA	Immunoperoxidase test
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
kV	Kilovolt
LB	Luria-Bertani
M	Molar
MA	Rhesus monkey kidney
Mab	Monoclonal antibody
Mg	Magnesium
ml	Millilitre
SPF	Specific-pathogen-free
Mg ₂ Cl	Magnesium chloride
Mg ₂ SO ₄	Magnesium sulphate
mM	Millimolar
MTP	Microtiter plate
uM	Micromolar
ug	Microgram
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDV	Newcastle disease virus
ng	Nanogram
nm	Nanometer
OD	Optical density
OPD	<i>O</i> -Phenylenediamine dihydrochloride
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
PD	Primer dimer
pi	Post inoculation
pmol	Picamol
PNT	Positive negative threshold
QGDPT	Quantitative gel diffusion precipitin test
RAPD	Random amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
RK	Rabbit kidney
RNA	Ribonucleic acid
RT	Reverse-transcriptase
SD	Standard deviation

SDS	Sodium dodecyl sulphate
S/P	Sample-to-positive ratio
SNT	Serum neutralization test
SPSS	Statistical program for social science
STC	Standard challenge strain
TAE	Tris-acetate-EDTA
TCVN	Tissue culture virus neutralization
TBE	Tris-borate-EDTA
TEM	Transmission electron microscopy
T _m	Melting temperature
TMB	Tetramethylbenzidine
Tris	2-amino-2-(hydroxymethyl)-1, 3 propandiol
UPGMA	Unweighted pair group with arithmetic mean
UPM	Universiti Putra Malaysia
UV	Ultraviolet
Vero	Green monkey kidney
VN	Virus neutralization
vv	Very virulent
VP	Virus protein
(w/v)	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Single/Three Letter Amino Acid Code		
Alanine	A	Ala
Asparagine	N	Asp
Aspartic Acid/Aspartate	D	Asp
Glutamine	Q	Glu
Glutamic acid/Glutamate	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val

CHAPTER 1

INTRODUCTION

Infectious bursal disease (IBD) is an acute highly contagious viral disease in chickens which is caused by IBD virus (IBDV). Lymphoid tissue is the primary target organ of IBDV with the special predilection for the bursa of Fabricius. The disease was first recognized as a clinical entity in 1957 in USA by Cosgrove (1962) and was referred to as 'avian nephrosis' because of the extreme kidney damage found in birds that succumbed to infections. Since the first outbreaks of IBD in the area of Gumboro, Delaware, "Gumboro disease" was a synonym for this disease and is still frequently used. The disease becomes important in poultry industry worldwide due to significant economic losses resulting from high mortality, impaired growth, excessive carcass condemnation and profound immunosuppression which lead to increase susceptibility to other pathogens. The disease can interfere the effectiveness of the vaccination programmes against other highly virulent disease (Allan, *et al.*, 1972; Okoye, 1984).

Two distinct serotypes of IBDV, designated as serotype 1 and serotype 2 have been identified. The serotype 1 strains are pathogenic to chickens and vary in their virulence, whereas serotype 2 strains, isolated from turkey are apathogenic to both turkey and chicken (Lukert and Saif, 1991). According to antigenic variation and virulence, serotype 1 strains can be divided into several groups: classical

virulent, attenuated, antigenic variant and very virulent (vv) strains (Cao, *et al.*, 1998).

Since the first outbreak of IBD in 1957, the disease was relatively under control due to proper immunization programmes in both the parent stock and their progeny. In the mid-80's, antigenic and pathogenic serotype 1, variants of IBDV were isolated by Saif and Rosenberger (Lukert, 1995) from the Delmarva area. Subsequently, the variants were recognized in other areas of the United States. The variant strains were significantly different antigenically to those of classical virulent IBDV and they could overcome higher levels of chicken maternal antibody within the first week to 10 days (Lukert, 1995). These variant strains were also different pathogenically which did not cause clinical disease in older chickens (4-6 weeks of age) when compared to the classical strains, but they could induce severe bursal atrophy and immunosuppression (Lukert, 1995). Recently, Giambrone (2001) described that the latest IBDV variants to evolve are strains that can contribute to infectious proventriculus. However, the evidence of the presence of the variant strains of IBD has never been presented in Europe or Malaysia.

In the late 1980's, outbreaks of clinical IBD with high mortality, up to 90% due to highly virulent strains of serotype 1 IBDV occurred throughout Europe (Chettle, *et al.*, 1989; Stuart, 1989; Van Den Berg, *et al.*, 1991). The disease had spread worldwide and was described in Asia in 1990's (Nunoya *et al.*,